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Lack of Inhibition of Intestinal Heme Oxygenase by Antibiotics and Tin-Protoporphyrin

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ABSTRACT. We assessed the *in vivo* and *in vitro* effects of antibiotics and tin-protoporphyrin (TP) on intestinal heme oxygenase (HO) activity using a gas chromatographic assay. This method measures the carbon monoxide produced from heme in the presence of NADPH. After *in vivo* administration of kanamycin (10 mg/kg body weight), ampicillin (200 mg/kg body weight) or neomycin (60 mg/kg body weight) with or without TP (65 μmol/kg body weight) to suckling rats, no significant difference in HO activity along the small intestine was observed. *In vitro* exposure of adult rat intestinal preparations to the antibiotics showed no significant decrease in HO activity between control and experimental tissue preparations. A concentration-dependent stimulatory effect of neomycin was observed. Subcutaneous administration of TP (25 μmol/kg body weight) to adult male Wistar rats revealed no significant inhibition of the intestine. However, *in vitro* addition of TP (12.5 μM) to the control tissue preparations of adult Wistar rats revealed highly significant inhibition in liver and spleen when compared to the unexposed control tissues. In contrast, when TP was added to control intestinal preparations no inhibition was observed. These findings suggest that suckling rat intestinal heme oxygenase is not inhibited by *in vivo* treatment with high concentrations of kanamycin, ampicillin, or neomycin. Furthermore, these antibiotics are not *in vitro* inhibitors of adult rat intestinal HO. Finally, adult rat intestinal HO is not inhibited either *in vivo* or *in vitro* by a concentration of TP that significantly inhibits liver and spleen activity. (*Pediatr Res* 23: 50–53, 1988)

Abbreviations

CO, carbon monoxide
TP, tin-protoporphyrin
HO, heme oxygenase
VeCO, excretion rate of CO

TP, a synthetic heme analog, has been reported to be a competitive inhibitor of HO (1–3). This rate-limiting enzyme in the heme catabolic pathway (4) catalyzes the equimolar formation of bilirubin and CO. *In vivo* studies with TP have demonstrated the drug's effectiveness in lowering serum bilirubin levels in rats (5), in mice with severe hemolytic anemia (6) in rhesus monkeys (7), and in man (8). Preliminary studies in our laboratory suggested that a single dose of TP has no suppressive effect on the excretion rate of CO (VeCO) in suckling rats (9, 10) unless the animals are first treated with broad spectrum antibiotics (11). Later studies, showing that heme is excreted into the bile of TP-treated adult rats (12, 13) pointed to two possible explanations for the paradox: 1) heme-degrading bacteria in the intestine produced CO (14), thus preventing us from observing the inhibitory effects of TP until the bacteria were eradicated by antibiotics or 2) antibiotics directly inhibited intestinal HO. Because heme reaching the intestine can be catabolized to bilirubin and CO by intestinal tissue HO (15), the question of inhibition of intestinal HO has potential clinical importance. In the present study, we assessed the *in vivo* and *in vitro* effect of antibiotics and TP on intestinal HO activity, using a gas chromatographic assay for measuring HO activity through the CO produced from heme in the presence of NADPH (16, 17).

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MATERIALS AND METHODS

Animals. Wistar rats were used for all experiments. For neonate studies, litters of rats were received within 12 h postpartum. The average weight of the suckling rats was 6.25 g. Adult males of 250–300 g each were used for adult experiments. Neonates were allowed free access to their mother between treatments and adults were fed MRH 22/5 Rodent Blox (Wayne Animal Diets, Chicago, IL) and water *ad libitum*. All animals were housed in a temperature-controlled room (24 ± 1° C) with a 12-h light cycle. All animals were sacrificed by decapitation.

TP. TP was purchased from Porphyrin Products Inc. (Logan, UT), and was prepared as described previously (11), stored in the dark and used within 1 h of preparation.

Methemalbumin. Two concentrations of methemalbumin were used for these experiments. A 2.0/0.15 mM concentration was used for *in vivo* antibiotic-treated suckling HO determinations (Table 1). Thirteen mg of hemin (Sigma Chemical Co., St. Louis, MO) were dissolved in 2.5 ml 0.4 M Na_3PO_4. Distilled water (6.5 ml) and 100 mg bovine serum albumin (Sigma) were added. The solution was gradually titrated to pH 7.4 with 10 N HCl under stirring. The volume was adjusted to 10 ml with distilled water and stored at 4°C for up to 1 month. A dilute (150/11.2 μM) methemalbumin solution was used for studies assessing *in vitro* antibiotic treatment of adult intestinal homogenates (Table 3) and *in vivo* and *in vitro* TP treatment of adult rats (Table 2). This solution was prepared by diluting the solution described above with 0.1 M KPO_4, pH 7.4 (buffer).

NADPH. NADPH (4.0 mg), tetrasodium salt, 8.5% water, type I (Sigma) was dissolved in 1.0 ml 0.1 M KPO_4 buffer, pH 7.4.

Antibiotics. The antibiotic solutions used for the *in vitro* incubations with adult rat tissue supernatants were prepared as follows. Twenty-five times concentrated solutions were made by dissolving 100 mg neomycin sulfate (The Upjohn Company, Kalamazoo, MI), 21.2 mg ampicillin sodium (Wyeth Laboratories, Philadelphia, PA), and 1.0 mg kanamycin sulfate (Beecham Laboratories, Bristol, TN) per ml of 0.1 M KPO_4 buffer, pH 7.4. These solutions were further diluted to 5× and 1× with the appropriate amount of buffer. Twenty μl of these solutions were

added to the reaction vials yielding final reaction medium concentrations (for the 25× solutions) of 25.0 mg neomycin/ml, 5.03 mg ampicillin/ml, and 0.25 mg kanamycin/ml. The antibiotics for the *in vivo* treatment of sucklings were similarly prepared in the following concentrations: 1.5 mg neomycin/ml, 250 mg ampicillin/ml, and 12.5 mg kanamycin/ml.

Tissue collection and preparation. Tissue supernatants were prepared for assay as follows.

Liver and Spleen. The organs were removed and washed with ice-cold buffer. Each organ was homogenized with a Biohomogenizer (Biospec Products, Inc, Bartlesville, OK) in 4 volumes of buffer and the preparations were centrifuged at 13,000 × *g* for 15 min. The supernatant was then analyzed.

Adult Intestine. The entire intestinal length was perfused with 20 ml of ice-cold buffer to remove the content. The intestine was then cut longitudinally into two equal sections. The mucosa was collected from one of the sections by gently scraping the tissue with a glass slide. The serosa was designated as what remained of the first section after the mucosa was removed. The second section was left intact and was referred to as "whole" in Tables 2 and 3. The three intestinal fractions were homogenized in 4 volumes of buffer and centrifuged as described above.

Neonate Intestine. After removal of the small intestine, the entire length was perfused with 5-ml of ice cold buffer. Three sections of 3 cm each were excised corresponding to the duodenum, jejunum, and ileum. Due to the fragile nature of the intestine, the mucosa could not be collected in the same manner as for the adult tissues. Instead, the intestinal segments were simply homogenized in 4 volumes of buffer and centrifuged as described above.

HO assay. Traditionally, *in vitro* HO activity is determined using a spectrophotometric method that measures bilirubin production (4). However, for this study, we used a gas chromatographic assay that measures the amount of CO produced from methemalbumin in the presence of NADPH (16–18). The procedure is a simplification of the method of Sunderman *et al.* (19). Twenty μl of the tissue preparation, 20 μl of the appropriate methemalbumin solution, and either 20 μl of NADPH (total) or buffer (blank) were placed in septum-sealed vials. For some studies, either antibiotics (20 μl) or TP (1.5 μl) was also added to vial. After a 5-min temperature equilibration, the vials were purged with CO-free air and allowed to incubate for 15 min at 37° C. The reaction was terminated by quick freezing the vials in a dry ice/acetone bath (−78° C). The CO produced in the vials was then quantified by a gas chromatograph coupled to a Reduction Gas Detector (Trace Analytical Inc., Menlo Park, CA) with a sensitivity of 1 pmol/CO vial (18, 20). CO production by total and blank, analyzed in duplicate, was calculated and HO activity is presented as the difference between total and blank reactions expressed as nmoles CO produced/h/mg protein.

As mentioned above, two concentrations of methemalbumin were used. Preliminary studies in our laboratory show that, due to competition, the effects of inhibitors such as TP may be reduced if the originally recommended (18) 2.0/0.15 mM methemalbumin solution is used for the assay. Inhibition is more clearly revealed when the less concentrated 150/11.2 μM solution is utilized.

Protein determination. The supernatant protein concentration was determined by the method of Lowry *et al.* (21). Bovine serum albumin (Sigma) was used as the standard.

Experimental procedure. In Vivo Antibiotic Treatment of Sucklings (Table 1). Upon arrival, Wistar rat litters were divided into three groups: saline-treated, antibiotic-treated, and TP + antibiotic treated. The treatment schedule closely followed that described by Posselt *et al.* (11). Antibiotic-treated and TP + antibiotic-treated pups were injected intramuscularly with 5 μl each of kanamycin sulfate (10 mg/kg body weight) and ampicillin sodium (200 mg/kg body weight) at t = 0, 16, 26, and 42 h. They were gavaged via Silastic tubing (0.064 × 0.030 cm ID, Dow Corning Corporation, Midland, MI) with 0.25 ml neomycin sulfate (60 mg/kg body weight) at t = 26 and 42 h. The saline-treated pups were injected and gavaged similarly with 0.9% NaCl. The TP + antibiotic-treated group also received a single subcutaneous injection of 40 μl of TP (65 μmol/kg) at t = 0 h. The rats were sacrificed at t = 48 h, when Posselt *et al.* (11) observed the greatest difference between the CO excretion rats of TP-treated and saline-treated pups.

In Vitro Antibiotic Studies of Adult Rat Tissues (Table 2). Mucosal, serosal, and whole intestinal supernatants were obtained as described above from untreated adult Wistar rats. Heme oxygenase activity determinations were carried out as described above with 20 μl of the appropriate antibiotic solution added to the reaction vial. The activities were compared with controls in which 20 μl buffer had been added to the reaction vial in place of the antibiotics.

In Vivo TP Treatment of Adult Rats (Table 3). Adult male Wistar rats were injected subcutaneously with either 25 μmol TP/kg body weight (TP) or an equal volume of saline (control) at t = 0 h. Rats were sacrificed at t = 16 h and tissue preparation was carried out as described above.

In Vitro TP Treatment of Adult Rat Tissues (Table 3). Tissue preparations from control rats were used for these *in vitro* studies of HO activity; 1.5 μl of a 0.5 mM TP solution were added to reaction vials containing supernatant, methemalbumin, and either buffer or NADPH. Heme oxygenase determinations were performed as described above.

Statistics. All values are expressed as mean ± SD. Student's *t* test for independent and paired samples was used for statistical analysis of results.

RESULTS

After *in vivo* treatment of suckling rats (Table 1), no significant difference in HO activity in any segment of the intestine was observed between the three treatment groups (saline control, antibiotics, and TP + antibiotics). The activity gradient observed for the control group was maintained after the treatments. *In vitro* treatment of adult rat intestine preparations with neomycin, ampicillin, and kanamycin (Table 2) showed no significant decrease in HO activity between control and treated groups. A concentration-dependent stimulation of HO activity in both mucosa and whole intestine was observed after addition of neomycin. No significant inhibition of the adult rat intestine was observed after either *in vivo* subcutaneous treatment with TP or *in vitro* exposure to TP (Table 3). However, adult rat liver HO activity was significantly inhibited after *in vivo* treatment with the same concentration, and both spleen and liver HO activities were significantly inhibited after *in vitro* exposure to the drug.

DISCUSSION

After *in vitro* addition of neomycin, ampicillin, and kanamycin to adult rat intestine preparations, no suppression of HO activity is observed. Our data also suggest that these antibiotics are not *in vivo* inhibitors of the suckling rat intestine. Posselt *et al.* (11)

Table 1. *HO activity in suckling rat intestine supernatants after in vivo treatment with broad-spectrum antibiotics or antibiotics and 65 μmol TP/kg body wt**

	HO activity (mean ± SD, nmol/h/mg protein)		
	Duodenum	Jejunum	Ileum
Saline ($n = 6$)	1.7 ± 0.9	2.8 ± 0.9	3.5 ± 0.9
Antibiotics ($n = 9$)	1.5 ± 0.3	2.1 ± 0.6	3.0 ± 0.9
Antibiotics + TP ($n = 9$)	1.5 ± 0.4	2.4 ± 0.6	3.0 ± 1.0

* This study was performed using the 2.0/0.15 mM methemalbumin solution. No significant difference existed between treatment groups within each intestinal segment.

Table 2. *HO activity of adult rat intestinal tissue 13,000 × g supernatants in the presence of various concentrations of three antibiotics**

	HO activity (mean ± SD, nmol CO/h/mg protein)			
	Control	1×	5×	25×
Neomycin				
Mucosa ($n = 3$)	0.58 ± 0.31	0.89 ± 0.22	1.65 ± 0.36†	2.84 ± 1.24†
Whole ($n = 3$)	0.37 ± 0.18	0.58 ± 0.12	1.00 ± 0.08‡	1.66 ± 0.28‡
Ampicillin				
Mucosa ($n = 3$)	0.58 ± 0.31	ND§	ND	0.73 ± 0.43
Whole ($n = 3$)	0.37 ± 0.18	ND	ND	0.46 ± 0.16
Kanamycin				
Mucosa ($n = 3$)	0.58 ± 0.31	ND	ND	0.58 ± 0.36
Whole ($n = 3$)	0.37 ± 0.18	ND	ND	0.37 ± 0.13

* The control contained no antibiotics. Antibiotic concentrations are described in "Materials and methods." This study was performed using the 150/11.2 μM methemalbumin solution. Unmarked values were not significantly different from control.

† $p < 0.05$ compared to control.

‡ $p < 0.01$ compared to control.

§ Not determined.

Table 3. *HO activity of various tissue 13,000 × g supernatants from adult male Wistar rats after 16 h of in vivo exposure to saline (control) or 25 μmole TP/kg body wt (TP)**

	HO activity (mean ± SD, nmol CO/h/mg protein)		
	Control	TP	Control + TP
Liver ($n = 6$)	0.31 ± 0.13	0.15 ± 0.04†	0.08 ± 0.07‡
Spleen ($n = 6$)	1.09 ± 0.15	0.95 ± 0.23	0.10 ± 0.10§
Whole IT ($n = 5$)	0.27 ± 0.10	0.31 ± 0.20	0.32 ± 0.14
Mucosa ($n = 5$)	0.30 ± 0.15	0.33 ± 0.28	0.31 ± 0.28
Serosa ($n = 6$)	0.20 ± 0.05	0.22 ± 0.12	0.19 ± 0.06

* The last column (control + TP) lists HO activity of control tissues incubated *in vitro* with 12.5 μM TP. This study was performed using the 150/11.2 μM methemalbumin solution. Unmarked values were not significantly different from control.

† $p < 0.05$ compared to control.

‡ $p < 0.01$ compared to control.

§ $p < 0.001$ compared to control.

were only able to demonstrate inhibitory effect of TP, as measured by the VeCO, by first treating suckling Wistar rats with broad-spectrum antibiotics. Because undegraded heme is excreted into the intestine with the bile (12, 13), two explanations were offered for the observed phenomenon. 1) The antibiotics had eradicated the heme-degrading, CO-producing bacteria (14) in the gut or 2) the antibiotics had directly inhibited intestinal HO. The present study, however, eliminates the possibility of intestinal HO inhibition by antibiotics. By their negative findings, both the *in vivo* and *in vitro* studies point to the reduction of aerobic bacterial colonization in the gut as the cause of the decrease in CO excretion after TP treatment of the animals receiving broad-spectrum antibiotics to suppress and/or eradicate their colonization. It appears that the use of antibiotics in the study by Posselt *et al.* (11) simply "unmasked" the inhibition by TP of HO in other organs such as the liver.

An unexpected interesting finding is that adult rat intestinal HO is not inhibited either *in vivo* or *in vitro* at a concentration of TP that significantly inhibits liver and spleen HO activity. Although the lack of inhibition of intestinal HO in the *in vivo* studies (9) taken by themselves might be explained by the fact that the TP concentration could be low when compared with other tissues, such as the liver, the lack of *in vitro* inhibition, requires another explanation. Maines *et al.* (22) have offered evidence for two forms of HO. The possibility exists that there is yet another form of HO, peculiar to the intestinal mucosa cells, which is not inhibitable by TP. The design of the previous studies by our laboratory showing decreased CO excretion after TP treatment in animals that had prophylactically received broad-spectrum antibiotics did not exclude some measurable contribution to the VeCO by intestinal HO degradation of heme, reaching the intestine via the bile (12, 13). However, the fact that the suppression of CO excretion could be appreciated only after antibiotics suggests that bacterial breakdown of heme was an important source of CO in animals not treated with antibiotics. This might be further interpreted as suggesting that a comparable amount of heme was not absorbed by the intestine, precluding intestinal heme oxygenase breakdown of heme. The possibility of heme breakdown by intestinal HO is important to consider because intestinal CO production from biliary heme could be coupled with bilirubin production by the HO in intestinal mucosal cells. This bilirubin could be absorbed into the circulation, thereby compromising the efficacy of TP for reducing serum bilirubin levels in neonates.

Because intestinal HO appears to be different in terms of its vulnerability to competitive inhibition by TP compared to the HO present in other tissues, we suggest that the disposition of biliary heme be carefully evaluated, especially in appropriate neonatal models, where the enterohepatic circulation is prominent in the transitional period after birth. Although even lower concentrations of TP than those used in these experiments may be effective at inhibiting HO in other tissues, such as the liver, heme diverted to the intestine could be degraded to CO and bilirubin because of the lack of HO inhibition in this tissue. In light of the presented results, oral administration of TP would not be an effective solution. The enteral route, however, with direct intestinal absorption of TP, might still represent a feasible option for achieving effective inhibitory concentrations of TP in other sensitive organs.

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